EXPLORING THE PROBIOTIC EFFECTS OF FOXTAIL MILLET AND BROWNTOP MILLET ON GUT MICROBIOTA HEALTH

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Keywords:

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Introduction:

The gut is one of the most densely inhabited microbial ecosystems. A sophisticated and incredibly varied population of microbes, including viruses, bacteria, archaea, and even parasites, are found there (Mu *et al.,* 2025). The human gastrointestinal tract processes about 60 tonnes of food over the course of a lifetime, in addition to a large number of environmental bacteria that pose a serious risk to gut integrity (Elizabeth and Nathalie, 2017). Approximately 150 times as many genes are present in the human gut microbiota as in the total human genome (Afzaal *et al.,* 2022). It is generally acknowledged that the human body contains over 100 trillion microorganisms, which play a crucial part in a number of biological processes, including health and illness. Due to its multidirectional and communicational connection or axis with other organs through neurological, endocrine, humoral, immunological, and metabolic pathways, the gut microbiota has lately been categorized as a “vital organ”. Numerous studies have substantiated the idea that gut microbiota is crucial in regulating immunity, energy balance, weight gain or loss, and diseases associated with obesity (Afzaal *et al.,* 2022).

Millets are ancient grains cultivated primarily in arid and semi-arid regions, serving as staple foods in parts of Asia and Africa (Jinu *et al.,* 2024). Millets typically

consist of 60-70% carbohydrates, 6-9% proteins, 10-12% fibres, 1-5% fats, and 2-4% minerals leading to their designation as “nutricereals” (Awasthi *et al.,* 2025). Beyond their nutritional value, millets contain bioactive compounds like flavonoids and phenolics, which contribute to their therapeutic properties. Regular consumption of millets has been associated with health benefits, including the management of lifestyle-related disorders such as diabetes, cardiovascular diseases, and certain cancers. Additionally, their gluten-free nature makes them suitable for individuals with celiac disease. The resilience of millets to harsh climatic conditions and their low water requirements further highlight their potential role in promoting sustainable agriculture and enhancing food security (Jinu *et al.,* 2024).

Types of millets

1. Foxtail Millet (Navane) - Daily intake of 50g significantly reduced fasting blood glucose and 2-hour postprandial glucose in subjects with impaired glucose tolerance over 6 weeks (Ren *et al.,* 2018).
2. Brown Top Millet (Korale) - Contains phenolic compounds with antioxidant properties that prevent oxidative DNA damage and inhibit enzymes related to carbohydrate digestion (Sunagar and Sreerama, 2023).
3. Kodo Millet (Araka) - Supplementation improved glucose tolerance and prevented increases in serum cholesterol and lipid parameters in high-fat diet-fed rats (Sarma *et al.,* 2017).
4. Barnyard Millet (Oodalu) - Consumption believed to offer health benefits against diabetes, cardiovascular diseases, and obesity due to its rich micronutrient and fiber content (Bhatt *et al.,* 2023).
5. Little Millet (Saame) - Rich in fiber, protein, and antioxidants, contributing to its *potential in managing diseases like cardiovascular issues and diabetes (Srilekha et al., 2019).*

FOXTAIL MILLET

Foxtail millet (*Setaria italica*) is among the oldest cultivated cereals, extensively grown in the arid and semi-arid regions of Asia and Africa. It is valued for its resilience to drought and adaptability to diverse soil conditions, making it a staple in areas with challenging agricultural environments (Bansal, 2023). Foxtail millet

has been associated with various health benefits, including hypolipidemic (lipid-lowering), low glycemic index, and antioxidant properties (Sharma and Niranjan, 2017). It is commonly known in India as Kangni (Hindi), Kang (Gujrati), Navane (Kannada), Kaon dana (Bengali), Kavalai, and Tenai (Tamil), Kangam (Oriya). Foxtail millet has a typical domesticated plant architectural form consisting of a single stalk or a few tillers, with large inflorescences that mature more or less at the same time (Singh et al., 2017).

BROWNTOP MILLET

Browntop millet (*Brachiaria ramosa*) is small-seeded annual grass cultivated as grain crop, primarily on the marginal lands in dry areas in temperate, subtropical and tropical regions. It contains phytochemicals such as flavonoids, quinones, tannins, and resin (Singh et al., 2022). Brown top millet is becoming more and more popular because of its remarkable nutritional profile and capacity to withstand hot, dry weather with little water or other inputs. The rain-fed states of Andhra Pradesh, Karnataka, and other areas of North-Central India are where it is primarily grown (https://www.myfitnesspal.com/food/calories/brown-top-millet-1087247439).

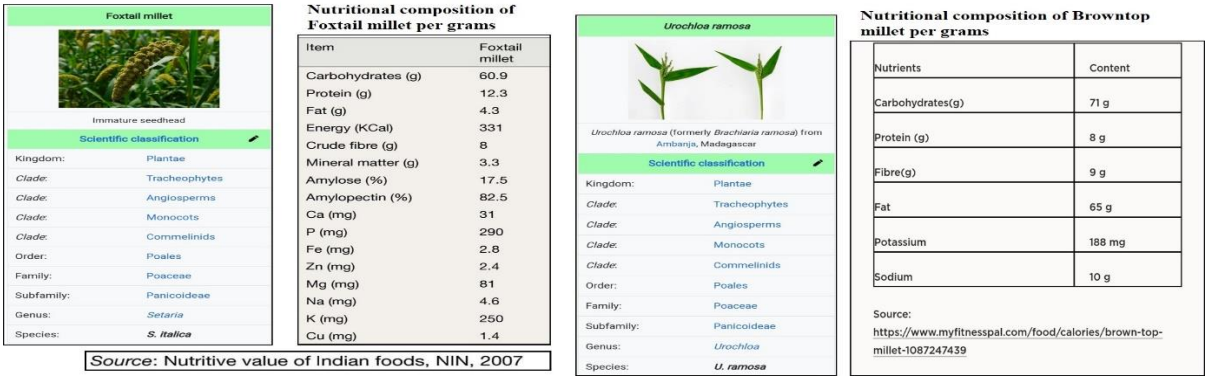


Figure 1: Foxtail Millet and Browntop Millet and their nutritional value

Figure 2: Illustrating Foxtail millet and Browntop Millet with sead coat and without seed coat

Objectives:

- To isolate different types of probiotics in Foxtail millet and Browntop millet.
- Identification of Probiotics.
- Biochemical characterization of probiotics.
    - Measuring of pH at different fermentation intervals.
    - Enzyme Assays – Amylase, Phosphatase, Esterase, Lipase, Peroxidase, Superoxide dismutase.
    - Protein Analysis by SDS-PAGE.
    - Antibacterial properties of probiotics.
    - Polyphenols estimation by FC method.
    - Analysis of polyphenols by FTIR.
    - Analysis of cytotoxic properties of polyphenols.
- Preparation of probiotics (Product).

Methodology:

- Fermentation Process: Millets were soaked, boiled, and fermented both with and without curd.
- Sample Screening: Post-fermentation samples were streaked on LB agar plates and incubated at 37°C.
- Probiotic Identification: Colonies were characterized morphologically and with Gram staining. 16S rRNA sequencing was used for identification.
-Biochemical Tests: pH of each probiotics was measured and Enzyme activities were determined (Amylase, Esterase and Phosphatase). Protein pattern will be analyzed by SDS-PAGE.
- Antibacterial Analysis of Probiotics: Effect of probiotics against pathogens like *S. aureus, S. marcescens,* and *P. rettgeri* was tested.
-Polyphenols of Millets: Polyphenol content estimated by FC method and Composition of Polyphenols was analysed by FTIR.
- Antibacterial Analysis of Polyphenols: Effect of polyphenols against pathogens like *S. aureus, S. marcescens,* and *P. rettgeri* was tested.
-Cytotoxic effect of Polyphenols: Will be analysed on Human Colorectal Carcinoma cell lines (HCT 116).
-Lyophilization: Isolated probiotic strains were freeze-dried to prepare probiotic formulations.

Results:

Probiotics Profiling

i. Soaking - Soak 1g of millet in 10mL of drinking water overnight (12-14 hours) in a mud pot.

ii. Boiling - Boil the soaked millets in low flame for 35-40 minutes. After cooling, the mouths of the mud pots were closed with a clean cotton cloth for fermentation without and with curd (1:1 ratio).

iii. With Curd sample - Add Onion, Green chilli, and Curry leaves, mix it thoroughly and incubate it for 6-8 hours.

Figure 3: Soaking of Foxtail millet and Browntop millet in mud pot

Figure 4: Illustration showing Foxtail millet and Browntop Millet after boiling

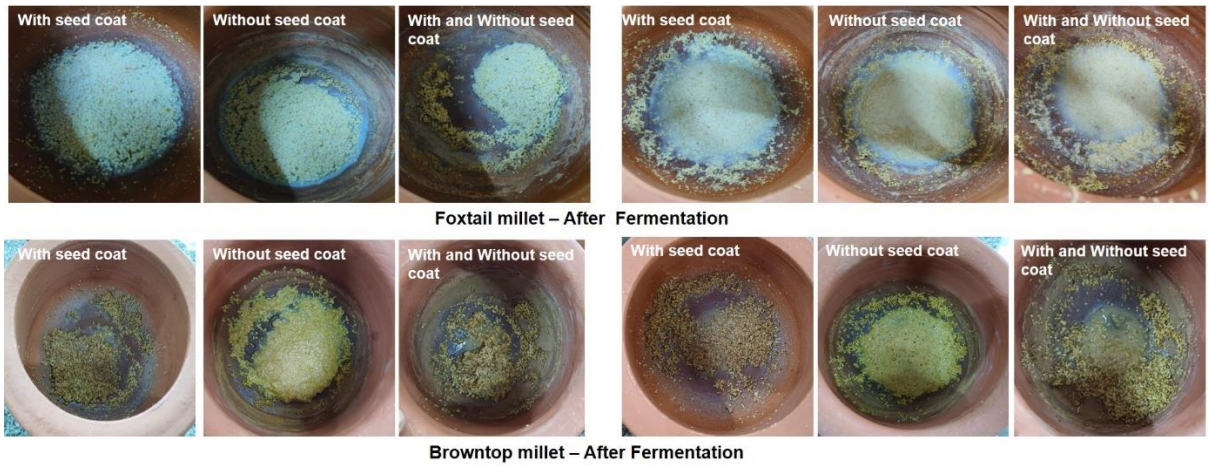


Figure 5: Illustration showing Foxtail millet and Browntop Millet after fermentation

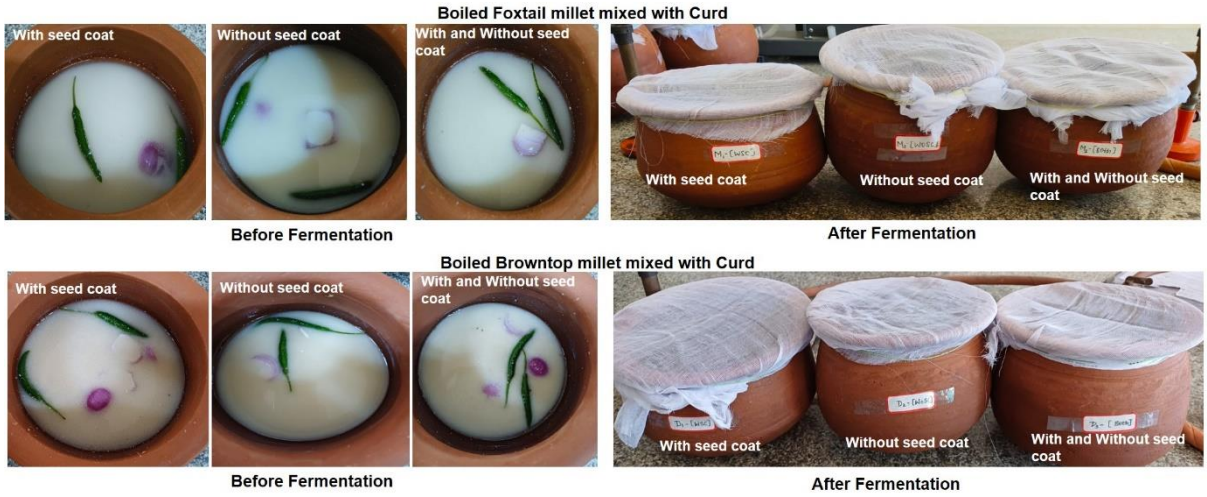


Figure 6: Illustration showing Boiled Foxtail millet and Browntop Millet were mixed with curd, green chilli, and onion

Screening of Probiotics: Mix the millet sample thoroughly after fermentation, the porridge and dilute it in autoclaved distilled water (10^{-1}-10^{-2}). Further streaked the samples on LB agar plates and incubate at 37°C.

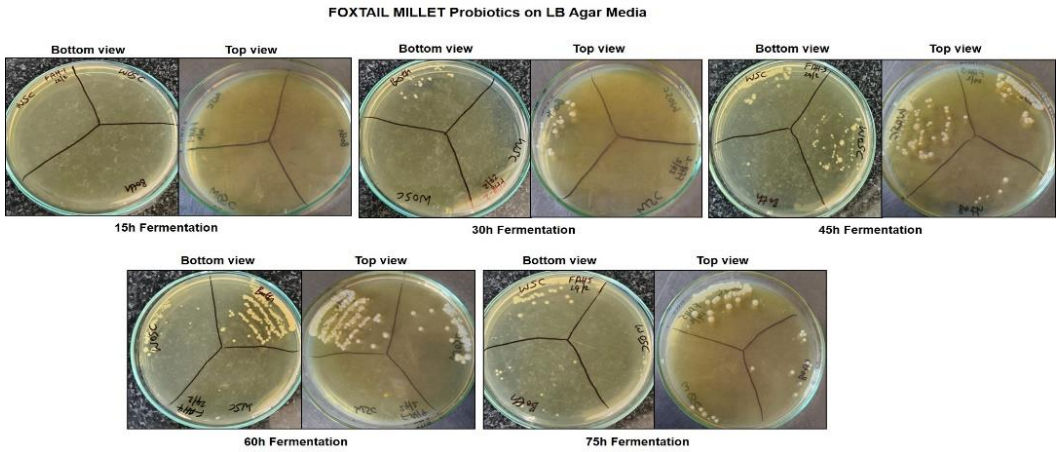


Figure 7: Illustration showing the Foxtail Millet Probiotics after incubation

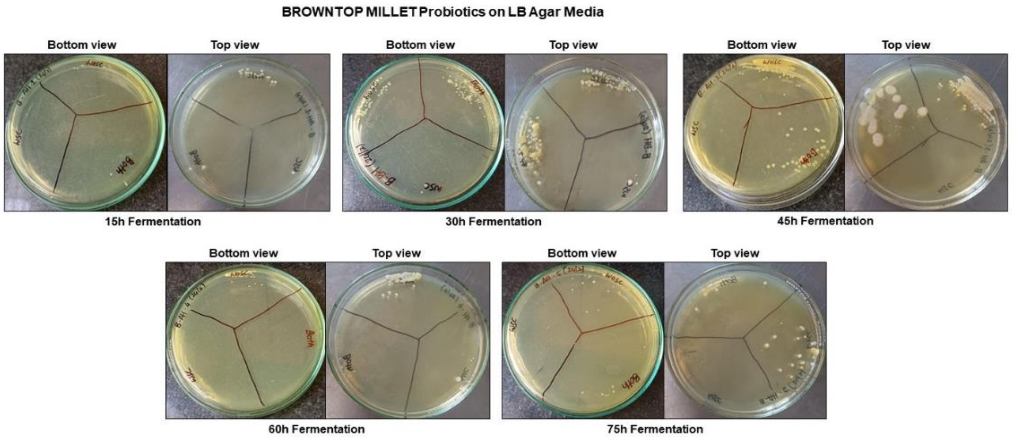


Figure 8: Illustration showing the Browntop Millet Probiotics after incubation

After incubation, in both Foxtail millet (FM) and Browntop millet (BTM) around 6-50 colonies were grown in each fermentation intervals. The individual colonies were sub cultured for purification and characterization.

Figure 9: Streaking of Individual colonies isolated from Foxtail Millet and Browntop millet on LB agar based on unique morphology

Identification of probiotics: Each bacterial species by regular Gram Staining based on colony morphology. Nearly 8 different probiotics were isolated and used for further characterization.

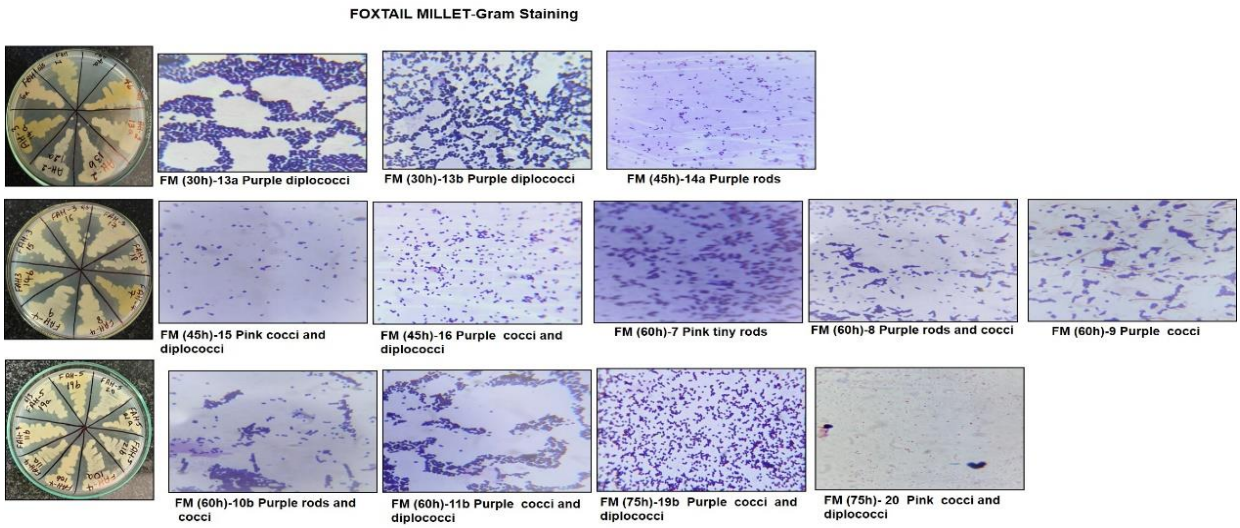


Figure 10: Microscopic visualization of stained samples of Foxtail Millet depending on Gram nature

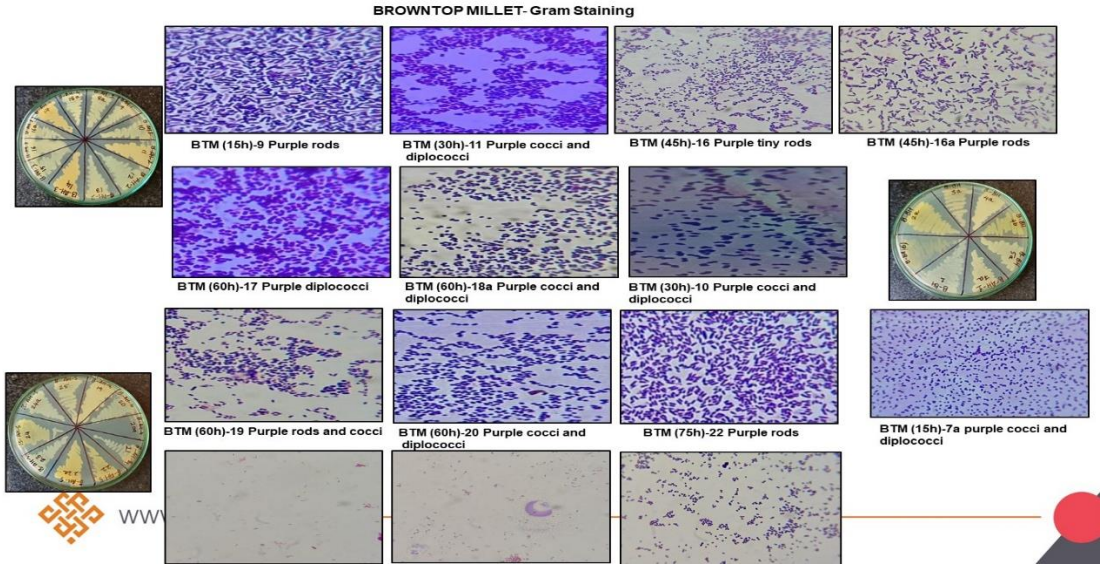


Figure 11: Microscopic visualization of stained samples of Browntop Millet depending on Gram nature

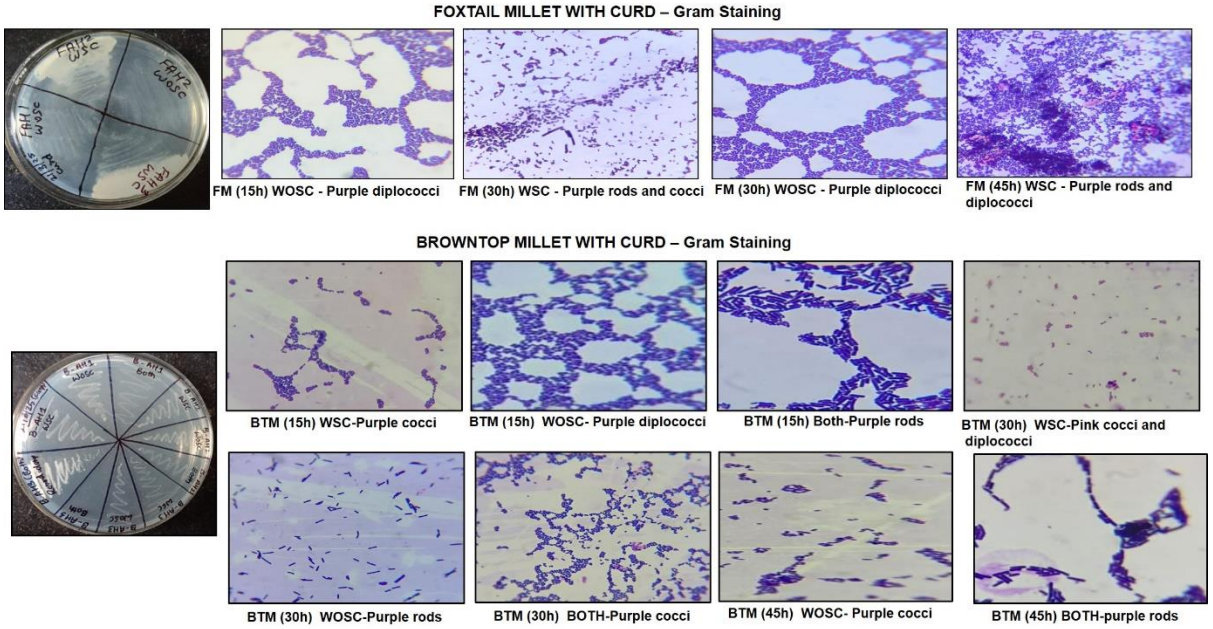


Figure 12: Microscopic visualization of stained samples of Foxtail Millet and Browntop Millets with curd

Nearly 6 different probiotics were isolated from Foxtail Millet and Browntop Millet fermented with curd and used for further characterization.

Different Types of Probiotics in Foxtail Millet without and with Curd - Based on Gram Staining

15h	30h	45h	60h	75h
	FM (30h)-13b Purple diplo - Cocci (BOTH)	FM (45h)-14a Purple rods (WSC)	FM (60h)-7 Pink rods (WSC)	FM (75h)-19b Purple cocci and Diplo-cocci (WSC)
		FM (45h)-16 Purple cocci and Diplo-cocci (BOTH)	FM (60h)-8 Purple rods and cocci (WOSC)	FM (75h)-20 Pink cocci and diplo-cocci (WOSC)
			FM (60h)-9 Purple cocci (WOSC)	
			FM (60h)-10b Purple rods and cocci (BOTH)	
	Total Number Of Probiotics: 1	**Total Number Of Probiotics: 3**	**Total Number Of Probiotics: 4**	**Total Number Of Probiotics: 4**
FM (15h)-WOSC Purple diplococci	FM (30h)-WSC Purple rods and cocci	FM (45h)-WSC Purple rods and diplococci		
	FM (30h)-WOSC Purple diplococci			
Total number of probiotics - 1	**Total number of probiotics - 3**	**Total number of probiotics - 2**		

Different Types of Probiotics In Browntop Millet without and with Curd -Based On Gram Staining

15h	30h	45h	60h	75h
BTM(15h)- 9 Purple rods (BOTH)	BTM(30h)-11Purple cocci and diplococci (WOSC)	BTM (45h)-16 Purple tiny rods (BOTH)	BTM(60h)-17 Purple diplococci (WSC)	BTM(75h)- 22 Purple rods (WOSC)
		BTM (45h)-16a Purple rods (BOTH)	BTM(60h)-18a Purple cocci and diplococci (WSC)	BTM(75h)- 22a Pink rods (WOSC)
			BTM(60h)-19 Purple rods and cocci (WOSC)	BTM(75h)-24 Pink cocci and diplococci (BOTH)
				BTM(75h)-24a Purple cocci and diplococci (BOTH)
Total number of strain : 1	**Total number of strains : 2**	**Total number of strains : 1**	**Total number of strains: 5**	**Total number of strains: 6**
BTM (15h)-WSC Purple cocci	BTM (30h)-WSC Pink cocci and diplococci	BTM(45h)-WOSC Purple cocci		
BTM (15h)-WOSC Purple diplococci	BTM (30h)-WOSC Purple rods	BTM (45h)-BOTH Purple rods		
BTM (15h)-BOTH Purple rods	BTM (30h)-BOTH Purple cocci			
Total number of strains:3	**Total number of strains:4**	**Total number of strains:2**		

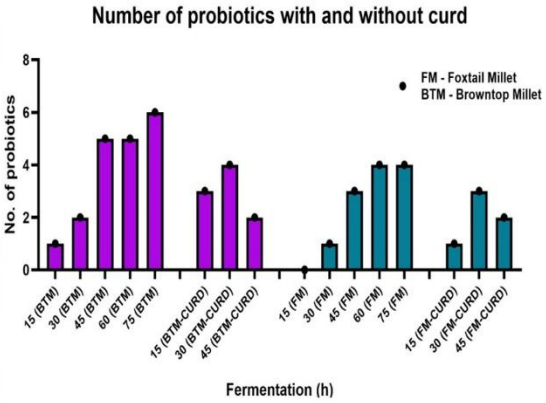


Table 1 and Figure 13: Types of probiotics in Foxtail Millet and Browntop Millet without and with curd and Graph showing number of Probiotics from Foxtail millet and Browntop millet based on Gram Staining.

Highest number of probiotics was observed at 45h and 60h fermentation without curd, especially in FM. The addition of curd reduced the number of probiotics in both FM and BTM. BTM showed a slightly higher probiotic count than FM overall, especially at 60h.

MORPHOLOGY	NUMBER OF STRAINS
Purple cocci (BTM)	0
Purple diplococci (BTM)	1
Purple cocci and diplo cocci (BTM)	5
Purple rods (BTM)	4
Purple rods and cocci (BTM)	1
Pink rods (BTM)	1
Pink cocci and diplococci (BTM)	1
Purple cocci (FM)	1
Purple diplococci (FM)	2
Purple cocci and diplo cocci (FM)	3
Purple rods (FM)	1
Purple rods and cocci (FM)	2
Pink rods (FM)	1
Pink cocci and diplococci (FM)	2

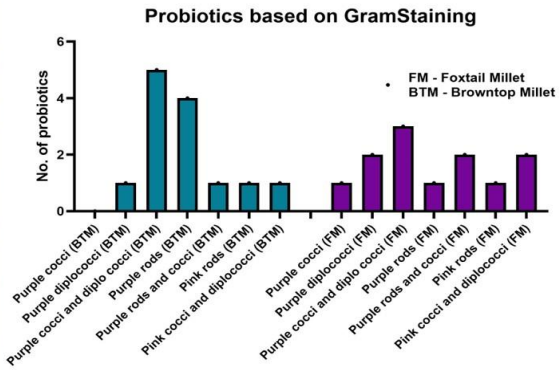


Table 2 and Figure 14: Types of probiotics in Foxtail Millet and Browntop Millet based on Gram Staining and Graph showing number of Probiotics from Foxtail millet and Browntop millet based on Gram Staining morphology.

BTM samples had a higher number of Probiotics with Gram-positive (purple) rods and cocci. Whereas FM showed a more balanced distribution of Probiotics with pink (Gram-negative) cocci and diplococci. Overall, BTM dominated in the number of Gram-positive probiotic forms.

Biochemical Characterization of probiotics

Measuring of pH at different fermentation intervals

FOXTAIL MILLET	pH	BROWNTOP MILLET	pH
FM(15h) WSC	8	BAH1 WSC	7
FM(15h) WOSC	8	BAH1 WOSC	7
FAH1 BOTH	7	BAH1 BOTH	8
FAH2 WSC	7	BAH2 WSC	7
FAH2 WOSC	8	BAH2 WOSC	7
FAH2 BOTH	7	BAH2 BOTH	8
FAH3 WSC	7	BAH3 WSC	7
FAH3 WOSC	7	BAH3 WOSC	7
FAH3 BOTH	7	BAH3 BOTH	8
FAH4 WSC	7	BAH4 WSC	7
FAH4 WOSC	7	BAH4 WOSC	6
FAH4 BOTH	7	BAH4 BOTH	8
FAH5 WSC	7	BAH5 WSC	7
FAH5 WOSC	7	BAH5 WOSC	7
FAH5 BOTH	7	BAH5 BOTH	7
FOXTAIL MILLET (CURD SAMPLE)	**pH**	**BROWNTOP MILLET (CURD SAMPLE)**	**pH**
FAH1 WSC	6	BAH1 WSC	7
FAH1 WOSC	6.5	BAH1 WOSC	6
FAH1 BOTH	6.5	BAH1 BOTH	6
FAH2 WSC	6	BAH2 WSC	6
FAH2 WOSC	6.5	BAH2 WOSC	6
FAH2 BOTH	6	BAH2 BOTH	6
FAH3 WSC	6	BAH3 WSC	6
FAH3 WOSC	6	BAH3 WOSC	6
FAH3 BOTH	6.5	BAH3 BOTH	6

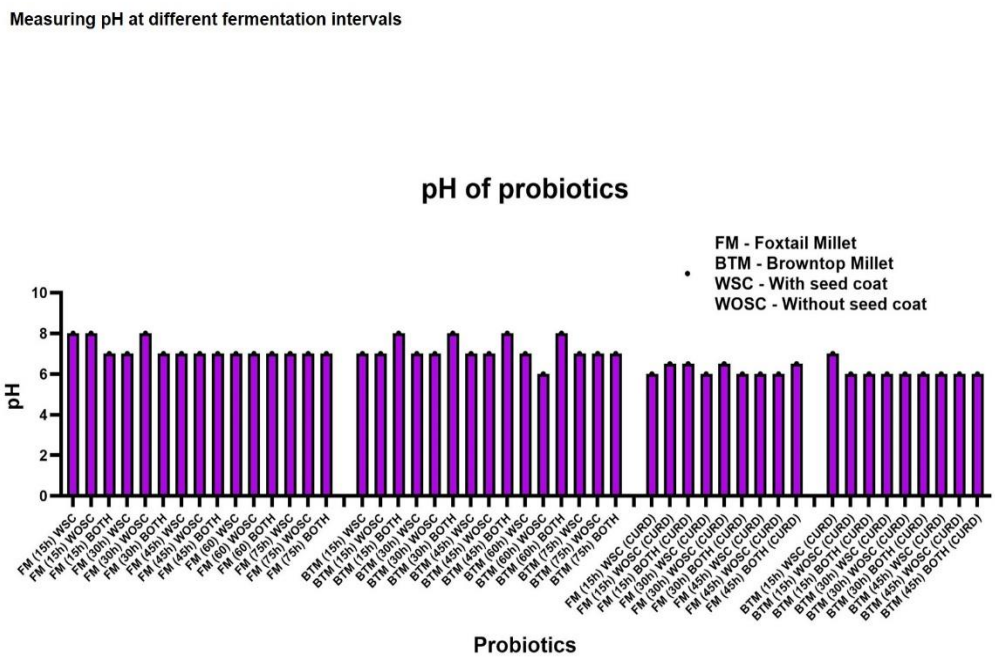


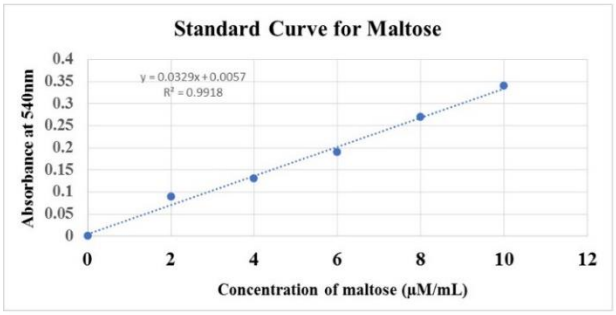
Table 3 and Figure 15: pH of probiotics and Graph showing pH of Probiotics of Foxtail millet and Browntop millet based on fermentation intervals.

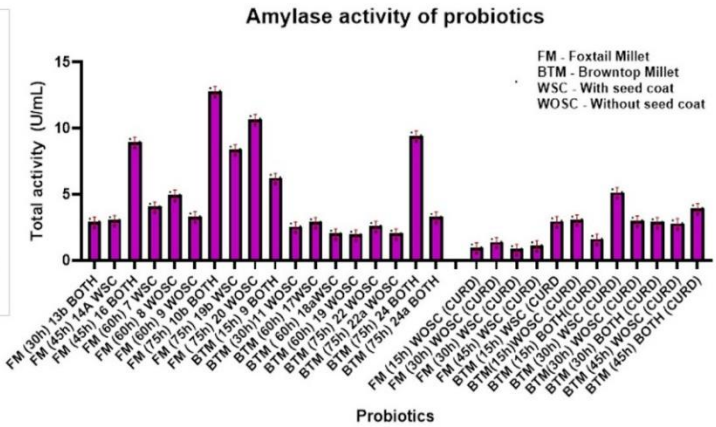
All the FM and BTM samples had a pH between 6 to 8. BTM (without seed coat) and FM (with seed coat) at longer fermentation intervals had biological pH values. CURD fermented samples maintained slightly lower pH compared to their non-CURD counterparts.

- **Enzyme Assay-Amylase**

Tabular Column for Maltose Estimation

Test tube no.	Conc. of std Maltose (umoles)	Volume of std Maltose (mL)	Volume of distilled water (mL)	Volume of DNS reagent (mL)	Heat in boiling water bath for 15mins and cool	Volume of distilled water (mL)	Absorbance at 540nm
Blank	0	0	0.20	0.1		1.7	0.00
1	2	0.04	0.16	0.1		1.7	0.099
2	4	0.08	0.12	0.1		1.7	0.164
3	6	0.12	0.08	0.1		1.7	0.27
4	8	0.16	0.04	0.1		1.7	0.317
5	10	0.20	0.00	0.1		1.7	0.151





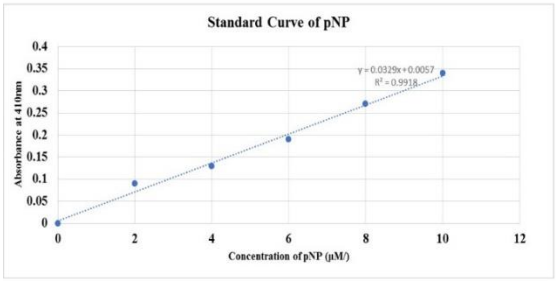
- Table 4 and Figure 16: Tabular column for Standard curve of Maltose and Amylase activity of Foxtail Millet and Browntop Millet probiotics.

Highest amylase activity was observed in Foxtail Millet and Browntop Millet with and without seed coat at 75h fermentation interval. Whereas in the case of CURD fermented samples, Amylase activity was low.

- **Enzyme Assay-Phosphatase**

Standard curve for pNP

Test tube no.	Conc. of std pNP (umoles)	Volume of std pNP(mL)	Volume od distilled water (mL)	Volume of NaOH (mL)	Volume of distilled water (mL)	Absorbance at 410nm
Blank	0	0	0.25	0.25	1.5	0.00
1	2	0.05	0.20	0.25	1.5	0.09
2	4	0.10	0.15	0.25	1.5	0.13
3	6	0.15	0.10	0.25	1.5	0.19
4	8	0.20	0.05	0.25	1.5	0.27
5	10	0.25	0.00	0.25	1.5	0.34



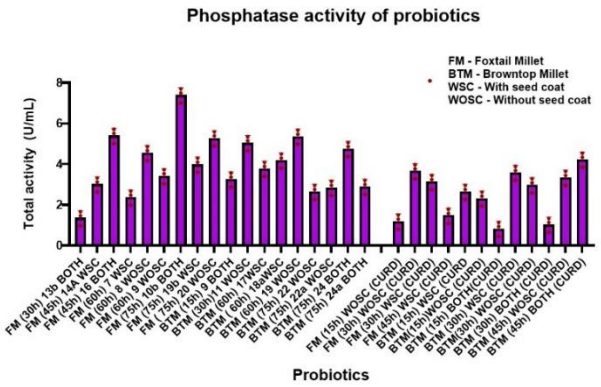


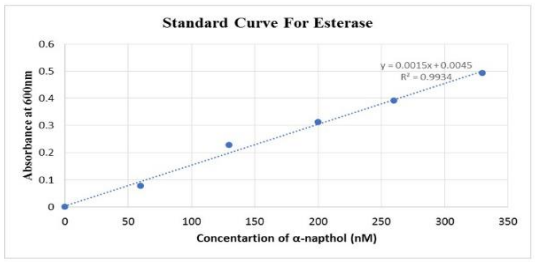
Table 5 and Figure 17: Tabular column for Standard curve of pNP and Phosphatase activity of Foxtail Millet and Browntop Millet probiotics.

Maximum Phosphatase activity was detected in Foxtail Millet and Browntop Millet with and without seed coat between 45 to 75h fermentation interval. CURD fermented samples showed reduced phosphatase activity across all combinations.

- **Enzyme Assay-Esterase**

Standard curve for Esterase

Test tube no.	Conc. of std α – Naphthol (ug/ul)	Volume of std α – Naphthol (mL)	Volume of Buffer (mL)	Volume of DBLS reagent (mL)	Incubate for 15mins	Absorbance at 600nm
Blank	0	0	1.67	0.33		0.00
1	50	0.06	1.61	0.33		0.078
2	100	0.13	1.54	0.33		0.227
3	150	0.20	1.47	0.33		0.312
4	200	0.26	1.41	0.33		0.392
5	250	0.33	1.34	0.33		0.492



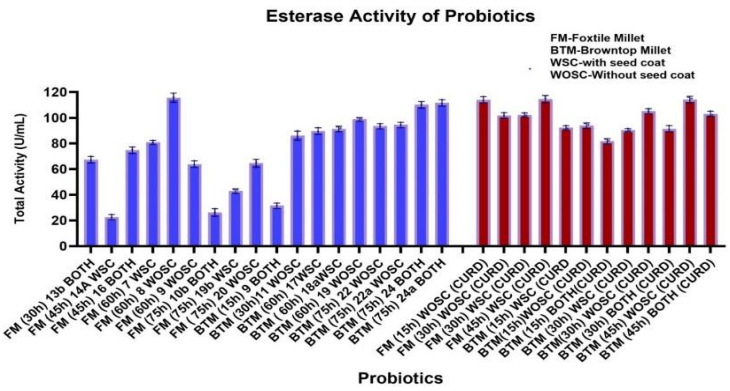


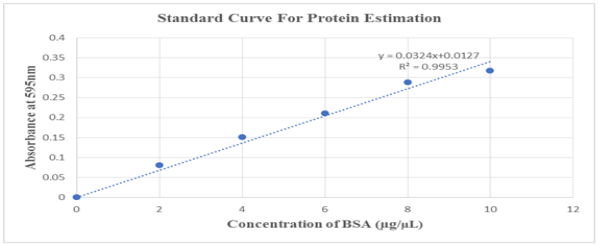
Table 6 and Figure 18: Tabular column for Standard curve of Esterase and Esterase activity of Foxtail Millet and Browntop Millet probiotics.

From the graph highest esterase activity was observed in Foxtail Millet from 45 to 60h and in Browntop Millet from 30 to 75h fermentation interval with and without seed coat (blue bars). In case of CURD fermented samples (red bars) both Foxtail millet and Browntop Millet, enzyme activity was maintained relatively constant from 15 to 45h fermentation intervals.

- Protein Estimation

Standard curve for Protein

Test tube no.	Conc. Of std Sol (ug/mL)	Volume of BSA(uL)	Volume od distilled water (uL)	Volume of Bradford's reagent(uL)	Absorbance at 595nm
Blank	0	0	100	500	0.00
1	2	2	98	500	0.081
2	4	4	96	500	0.151
3	6	6	94	500	0.210
4	8	8	92	500	0.288
5	10	10	90	500	0.317



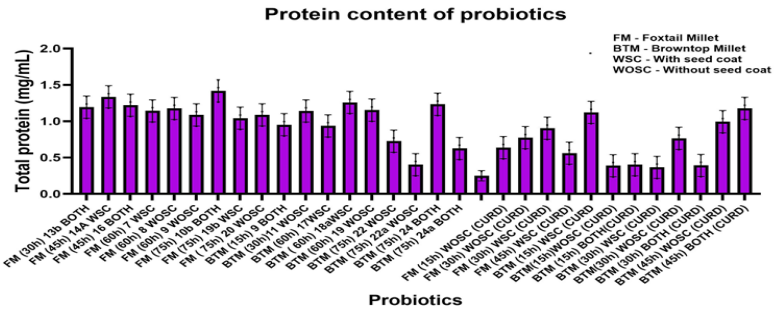
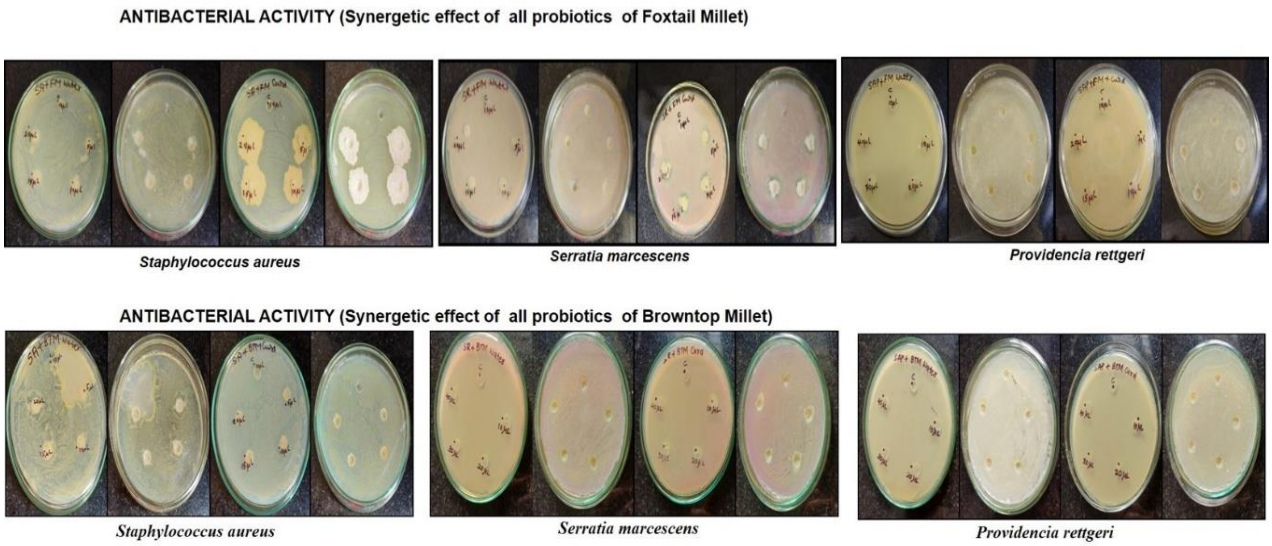


Table 7 and Figure 19: Tabular column for Standard curve of Protein and Estimation of Protein content in Foxtail Millet and Browntop Millet probiotics.

From the above result highest protein content in Foxtail Millet and Browntop Millet was observed between 1 to 1.5mg/mL from 30 to 75h fermentation intervals. Whereas in the case of CURD fermented samples the protein concentrations were maintained same as without curd samples at fermentation intervals of 15 to 45h. Samples with seed coat (WSC) yielded higher protein levels compared to those without (WOSC).

Antibacterial properties of probiotics (Synergistic effect of all probiotics of Foxtail Millet and Browntop Millet)



ZONE OF INHIBITION OF PROBIOTICS ON PATHOGENIC BACTERIA	DIAMETER (cm)
Staphylococcus aureus + Foxtail millet probiotics	5uL-0.2cm, 10uL-0.2cm, 15uL-0.3cm, 20uL-0.4cm
Staphylococcus aureus + Foxtail millet probiotics with curd	5uL-0.6cm, 10uL-0.8cm, 15uL-0.9cm, 20uL-1.0cm
Serratia marcescens + Foxtail millet probiotics	5uL-0.2cm, 10uL-0.3cm, 15uL-0.3cm, 20uL-0.3cm
Serratia marcescens + Foxtail millet probiotics with curd	5uL-0.5cm, 10uL-0.6cm, 15uL-0.8cm, 20uL-1cm
Providencia rettgeri + Foxtail millet probiotics	-
Providencia rettgeri + Foxtail millet probiotics with curd	5uL – 0.5cm, 10uL – 0.6cm, 15uL – 0.8cm, 20uL – 1.0cm
Staphylococcus aureus + Browntop millet probiotics	5uL-0.2cm, 10uL-0.3cm, 15uL-0.3cm, 20uL-0.4cm
Staphylococcus aureus + Browntop millet probiotics with curd	5uL-0.2cm, 10uL-0.2cm, 15uL-0.3cm, 20uL-0.4cm
Serratia marcescens + Browntop millet probiotics	10uL-0.6cm, 20uL-00, 30uL-0.6cm, 40uL-00
Serratia marcescens + Browntop millet probiotics with curd	10uL-0.5cm, 20uL-0.7cm, 30uL-0.8cm, 40uL-0.9cm
Providencia rettgeri + Browntop millet probiotics	-
Providencia rettgeri + Browntop millet probiotics with curd	-

Figure 20 and Table 8: Antibacterial activity of Foxtail Millet and Browntop Millet showing Zone of Inhibition

Foxtail Millet and Browntop Millet probiotics fermented with curd showed better inhibition zones than without curd.

Polyphenols extraction

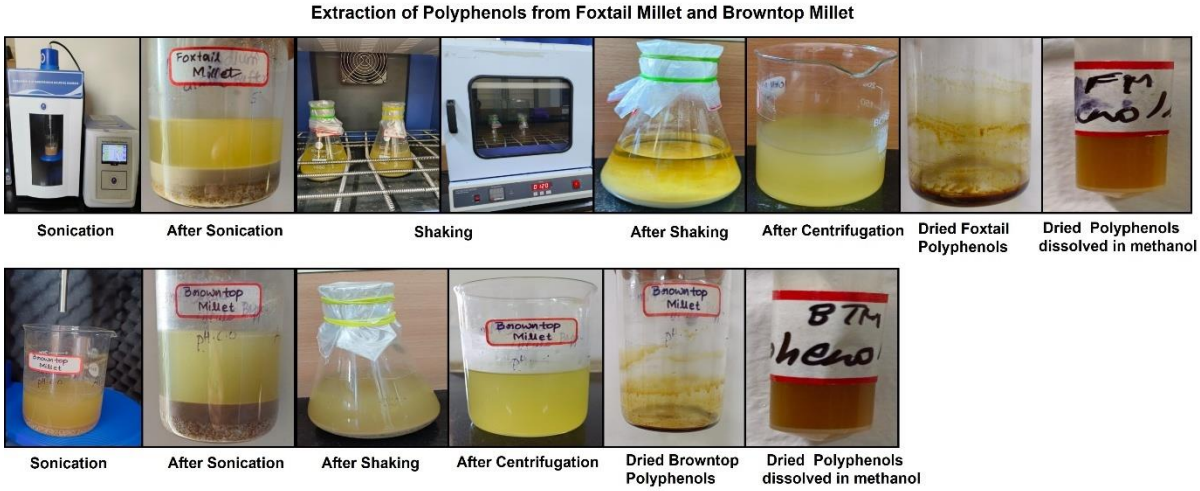
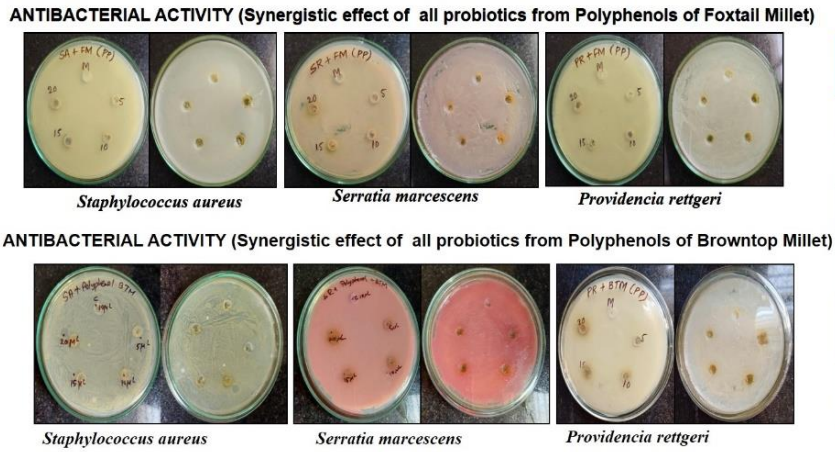


Figure 21: Polyphenol extraction of Foxtail Millet and Browntop Millet.

Estimation of Composition of Polyphenols: Composition was analyzed by FTIR (Fourier Transform Infrared Spectroscopy)



ZONE OF INHIBITION OF PROBIOTICS ON PATHOGENIC BACTERIA	DIAMETER (cm)
Staphylococcus aureus + *Foxtail millet* Polyphenol	13.4ug-0.2cm, 26.9ug-0.2cm 40.4ug-0.3cm, 53.9ug-0.4cm
Serratia marcescens + *Foxtail millet* Polyphenol	13.4ug-0.2cm, 26.9ug-0.2cm 40.4ug-0.3cm, 53.9ug-0.3cm
Providencia rettgeri + *Foxtail millet* Polyphenol	13.4ug-0.2cm, 26.9ug-0.2cm 40.4ug-0.3cm, 53.9ug-0.4cm
Staphylococcus aureus + *Browntop millet* Polyhenol	39.9ug-0.2cm, 79.8ug-0.2cm 119.8ug-0.3cm,159.7ug-0.3cm
Serratia marcescens + *Browntop millet* Polyphenol	39.9ug-0.1cm, 79.8ug-0.2cm 119.8ug-0.2cm, 159.7ug-0.3cm
Providencia rettgeri + *Browntop millet* Polyphenol	39.9ug-0.2cm, 79.8ug-0.3cm 119.8ug-0.3cm, 159.7ug-0.4cm

Figure 22 and Table 9: Antibacterial activity of Polyphenols from Foxtail Millet and Browntop Millet showing Zone of Inhibition.

In all three pathogenic bacterias (Staphylococcus, Serratia, Providencia), Foxtail millet polyphenols were effective but had smaller inhibition zones (maximum up to 53.9 µg – 0.4 cm), Whereas Browntop millet polyphenols showed the strongest antibacterial activity, with large inhibition zones up to 159.7 µg – 0.4 cm.

- Identification of each bacterial species by 16S rRNA sequencing (under progress)
- Enzymes lipase, Superoxide dismutase, Peroxidase (under progress)
- Protein pattern of Probiotics by SDS-PAGE (under progress)
- Cytotoxic properties of polyphenols (under progress)

Conclusions:

Both Foxtail Millet and Browntop Millet fermented samples showed a diverse range of Gram-positive probiotics such as rods, cocci, and diplococci. Significant enzymatic activity was observed, with active production of amylase and phosphatase. Polyphenol content was substantial, indicating antioxidant capacity. Probiotic extracts inhibited the growth of multiple pathogens, highlighting their antimicrobial potential. The overall findings suggest that these millets have high potential for functional food applications supporting gut health.

Future Scope:

1. Conduct human clinical trials to evaluate long-term probiotic benefits.
2. Develop millet-based probiotic products like drinks, capsules, or porridges.
3. Study molecular mechanisms of probiotic-microbiota interaction.
4. Analyze individual polyphenols for targeted health benefits.
5. Optimize fermentation conditions for higher probiotic yield.
6. Scale up fermentation and lyophilization for commercial use.
7. Promote millets as sustainable crops in agriculture.
8. Explore use in the nutraceutical industry for gut health supplements.
9. Extend research to other millet varieties for probiotic potential.
10. Investigate millet probiotics in managing gut-related disorders.